Regulation of NLRP3 and AIM2 inflammasome gene expression levels in gingival fibroblasts by oral biofilms

Bostanci, N ; Meier, A ; Guggenheim, B ; Belibasakis, G N

Abstract: Periodontal disease is an inflammatory condition that destroys the tooth-supporting tissues. The inflammation is initiated by oral bacteria in the form of multi-species biofilms, and is dominated by cytokines of the IL-1 family. IL-1 activation and processing is regulated by Caspase-1, within intracellular protein complexes, known as "inflammasomes". The present study employed culture supernatants of in vitro supragingival and subgingival biofilms, to challenge human GF cultures for 6h. The gene expression of inflammasome complex components was investigated by TaqMan qPCR. NLRP1 expression was not affected, whereas NLRP2 was not expressed. Supragingival biofilm challenge increased the expression of Caspase-1, the adaptor ASC, AIM2, as well as IL-1 and IL-18, but did not affect NLRP3 expression. Subgingival biofilm challenge enhanced Caspase-1, ASC, AIM2, IL-1 and IL-18 gene expression at lower concentrations, followed by their down-regulation at higher concentrations, which was also evident for NLRP3 expression. Hence, supragingival and subgingival biofilms differentially regulate the gene expressions of NLRP3 and AIM2 inflammasomes and their down-stream IL-1 targets. Increased inflammasome transcription in response to supragingival biofilms is commensurate with early inflammatory events in periodontal disease, whereas decreased transcription in response to subgingival biofilms corroborates the dampening of host immune responses, in favour of pathogen survival and persistence.

DOI: https://doi.org/10.1016/j.cellimm.2011.04.002

Regulation of NLRP3 and AIM2 inflammasome gene expression levels in gingival fibroblasts by oral biofilms

Nagihan Bostanci [a], Andre Meier [b], Bernhard Guggenheim [b], Georgios N. Belibasakis [b*]

[a] Oral Translational Research, [b] Oral Microbiology and Immunology, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Plattenstrasse 11, 8032 Zürich, Switzerland

** Corresponding Author:*
Dr. Georgios N. Belibasakis
Institute of Oral Biology
Center of Dental Medicine
University of Zürich
Plattenstrasse 11
8032 Zürich
Switzerland
e-mail: george.belibasakis@zzm.uzh.ch
Tel: +41-44-6343396
Fax: +41-44-6343310

Abbreviations

GF: gingival fibroblasts, NLR: nucleotide-binding oligomerization domain-like receptor, AIM2: Absent In Melanoma 2; ASC: apoptotic speck protein containing a C-terminal caspase recruitment domain; qPCR: quantitative real-time polymerase chain reaction, IL-1: interleukin-1

Abstract

Periodontal disease is an inflammatory condition that destroys the tooth-supporting tissues. The inflammation is initiated by oral bacteria in the form of multi-species biofilms, and is dominated by cytokines of the IL-1 family. IL-1 activation and processing is regulated by Caspase-1, within intracellular protein complexes, known as "inflammasomes". The present study employed culture supernatants of *in vitro* supragingival and subgingival biofilms, to challenge human GF cultures for 6 h. The gene expression of inflammasome complex components was investigated by TaqMan qPCR. NLRP1 expression was not affected, whereas NLRP2 was not expressed. Supragingival biofilm challenge increased the expression of Caspase-1, the adaptor ASC, AIM2, as well as IL-1β and IL-18, but did not affect NLRP3 expression. Subgingival biofilm challenge enhanced Caspase-1, ASC, AIM2, IL-1β and IL-18 gene expression at lower concentrations, followed by their down-regulation at higher concentrations, which was also evident for NLRP3 expression. Hence, supragingival and subgingival biofilms differentially regulate the gene expressions of NLRP3 and AIM2 inflammasomes and their down-stream IL-1 targets. Increased inflammasome transcription in response to supragingival biofilms is commensurate with early inflammatory events in periodontal disease, whereas decreased transcription in response to subgingival biofilms corroborates the dampening of host immune responses, in favour of pathogen survival and persistence.

Keywords: Oral biofilms; gingival fibroblasts; periodontal diseases; inflammasomes; Interleukin-1β; Interleukin-18; NLRP3; AIM2

1. Introduction

Periodontal diseases are the most common chronic inflammatory diseases in man. They are characterised by the inflammatory destruction of the tooth supporting (periodontal) tissues, eventually leading to tooth loss if left untreated. The etiological factor of this cluster of diseases is oral bacteria colonizing the tooth surfaces as polymicrobial biofilm communities [1]. Biofilms interact with the surrounding tissues periodontal tissues, triggering immune responses that result in tissue-destructive inflammation. Depending on the localization of the biofilm in relation to the gingival margin, this can be either “supragingival” (above) or “subgingival” (below) [2]. Bacterial products released by the biofilms can cause an inflammatory response by the periodontal tissues, aiming to eliminate the bacterial challenge [3]. However, rather than being protective, an excessive inflammatory response induces periodontal tissue damage [4]. Gingivitis is a form of periodontal disease in which the inflammatory response is restricted to the superficial gingival tissue, and is typically associated with the presence of a supragingival biofilm. It may progress to periodontitis, which involves further the inflammatory destruction of the tooth-supporting alveolar bone and interconnecting periodontal ligament, attributed mainly to highly virulent subgingival biofilms [1, 2, 5, 6]. Cytokines of the Interleukin(IL)-1 family, including IL-1β and IL-18, are central to the inflammatory host response in periodontal diseases [7, 8]. High IL-1β levels are detected in the gingival crevicular fluid [9] or gingival tissues [10], of patients with periodontal disease.

Inflammasomes are oligomeric molecular platforms that trigger the maturation of IL-1 cytokines in response to infection or stress signals at the cellular level, in order to engage them into innate immune defences [11]. The most well characterised inflammasomes are of the nucleotide-binding oligomerization domain-like receptor

(NLR). They are essentially intracellular pattern recognition receptors (PRRs) that detect pathogen-associated or danger-associated molecular patterns (PAMPs and DAMPs). The recognition of PAMPs or DAMPs by NLRs leads to the recruitment and activation of cysteine proteinase Caspase-1. In turn, Caspase-1 proteolytically activates the intracellularly stored pro-IL-1β into its mature secreted form, so that it can exert its pro-inflammatory properties. The NLRP3 inflammasome in particular, consists of three components, namely the NLRP3 scaffold, Caspase-1 and apoptotic speck protein containing a C-terminal caspase recruitment domain (ASC), the adaptor molecule that mediates the interaction of the former two. This inflammasome is activated in response to cell stress signals [12], bacteria and viruses, or their virulence factors [13-16], and is likely to play a key role in inflammatory periodontal disease [10]. Less information is currently available on NLRP2, but it is considered to be an inhibitor of the NLRP3-ASC interaction [17, 18]. The NLRP1 inflammasome is structurally different to NLRP3, as it does not require the mediation of ASC for the activation of Caspase-1 [19], and it can also activate Caspase-5 [20]. So far, this inflammasome is shown to be activated by the anthrax lethal toxin, causing cell death [21]. Absent In Melanoma (AIM)2 is a recently discovered non-NLR inflammasome that senses cytosolic double-stranded DNA, consequently inducing Caspase-1-dependent IL-1β maturation [22]. It is activated by a broad spectrum of DNA sources, such as bacteria, viruses, or even the host. A shared feature with the NLRP3 inflammasome is that the AIM2 platform also consists of ASC and Caspase-1 [23].

The gingival connective tissue has an important role in the protection and homeostasis of the periodontium. The main cell population of this tissue are the gingival (GF), synthesizing its collagenous matrix, but also responding to bacterial challenge by producing mediators of inflammation [24]. Non-immune cells, such as

GF, can recognize danger signals and initiate inflammatory responses in anatomical compartments less accessible to myeloid cells, thus fulfilling tasks usually performed by residential macrophages [25]. Although GF are less potent than macrophages in inflammatory cytokine production, their inflammasome expression could still regulate local host responses in the periodontium. To this extent, synovial fibroblasts are shown to express NLRP3 in models related to rheumatoid arthritis [26, 27].

Neither the expression of inflammasomes, nor their potential responses to polymicrobial oral biofilms have been investigated in GF. The present *in vitro* study aims to compare the capacity of a supragingival and a subgingival biofilm model to regulate the gene expression of various inflammasome components in human GF cultures.

2. Materials and Methods

2.1 *In vitro* biofilm model

The 6-species suprgagingival Zürich biofilm model [28] used in this study consisted of *Veillonella dispar* ATCC 17748 (OMZ 493), *Fusobacterium nucleatum* KP-F8 (OMZ 598), *Streptococcus oralis* SK248 (OMZ 607), *Actinomyces naeslundii* (OMZ 745)*, Streptococcus mutans* UAB159 (OMZ 918) and *Candida albicans* (OMZ 110).
The 10-species subgingival Zürich biofilm model [29] used in this study consisted of *Campylobacter rectus* (OMZ 697), *F. nucleatum* subsp. *vincentii* KP-F2 (OMZ 596), *Porphyromonas gingivalis* ATCC 33277T (OMZ 925), *Prevotella intermedia* ATCC 25611T (OMZ 278), *Tannerella forsythia* OMZ 1047, *Treponema denticola* ATCC 35405T (OMZ 661), *V. dispar* ATCC 17748T (OMZ 493), *A. naeslundii* OMZ 745, *S. intermedius* ATCC 27335 (OMZ 512), and *S. oralis* SK 248 (OMZ 607). Briefly, the supragingival or subgingival biofilms were grown in 24-well cell culture plates on

sintered hydroxyapatite discs, resembling natural tooth surfaces, and were pre-conditioned for pellicle formation with human mixed saliva for 4 h. To initiate biofilm formation, hydroxyapatite discs were covered for 16.5 h with 1.6 ml of growth medium consisting of 60% saliva, 10% human serum (pooled from three donors), 30% FUM culture medium [30] and 200 µl of a bacterial cell suspension containing equal volumes and density from each strain. After 16.5 h of anaerobic incubation at 37°C, the inoculum suspension was removed from the discs by 'dip-washing' using forceps, transferred into wells with fresh medium (60% saliva, 10% human serum, 30% FUM), and incubated for further 48 h in anaerobic atmosphere. During this time-period, the discs were dip-washed 3x and given fresh medium once daily. After a total 64.5 h of incubation, the biofilm supernatants were collected, filtered and stored at -80°C. The bacterial protein concentration in these supernatants was determined by the BCA Protein Assay (Pierce). For the experiments, these biofilm supernatant preparations were diluted into the final cell culture medium and maintained in the cell culture for up to 6 h [29]. Their concentration is expressed as total protein (µg/ml) present in the cell cultures.

2.2 Cell cultures

Primary human GF cell lines were established as previously described [31]. Briefly, gingival tissue biopsies used were obtained from healthy young individuals, who had their first premolar removed during the course of orthodontic treatment. Ethical approval was granted by the Human Studies Ethical Committee of Umeå University, Sweden, and informed consent was given by the subject. The cells were passaged and cultured in Minimum Essential Medium Alpha (Gibco), supplemented with 5 % heat-inactivated foetal bovine serum (Sigma), 50 U/ml penicillin, and 50 µg/ml

streptomycin (Sigma). For the experiments, GF cells at passage 3 were seeded at concentration 10 x 10^3 cells/cm^2 in antibiotics-free and 5% FBS culture medium, and were allowed to attach overnight, maintaining a sub-confluent status. Thereafter, the cells were cultured for 6 h in the presence or absence of ascending protein concentrations of biofilm supernatants.

2.3 Cytotoxicity assay

The GF cultures were exposed for 6 h to ascending protein concentrations of supernatants from the two biofilms and potential cytotoxicity was evaluated using the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega).

2.4 RNA extraction and cDNA synthesis

After completion of the experiments, the culture supernatants were removed from the culture and the cell monolayers were washed twice in PBS before being lysed. The collected cell lysate was homogenized with QIAshredder (QIAGEN), and total RNA was extracted by using the RNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions. RNA concentration was measured by a NanoDrop spectrophotometer. One μg of total RNA was then reverse transcribed into single-stranded cDNA by using M-MLV Reverse Transcriptase, $Oligo(dT)_{15}$ Primers, and PCR Nucleotide Mix according to the manufacturer's protocol (all from Promega), at 40°C for 60 min, and 70°C for 15 min. The resulting cDNA was stored at -20°C.

2.5 Quantitative real-time Polymerase Chain Reaction (qPCR)

For gene expression analyses, qPCR was performed in an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems). 18S rRNA was used as

endogenous RNA control in the samples (house-keeping gene). For the amplification reactions, the TaqMan Gene Expression Master Mix and Gene Expression Assay kits from Applied Biosystems were used (assay IDs NLRP1: Hs00248187-m1 , NLRP2: Hs001546938-m1, NLRP3: Hs00918085-m1, ASC: Hs01547324-m1, Caspase-1: Hs00354836-m1, AIM2: Hs00915710-m1, IL-1β: Hs00174097-m1, IL-18: Hs01038787-m1, and 18S rRNA: Hs99999901-s1). The standard PCR conditions were 10 min at 95ºC, followed 40 cycles at 95ºC for 15 seconds and 60ºC for 1 min. The expression levels of the target transcripts in each sample were calculated by the comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization to 18S rRNA.

2.6 Statistical analysis

A two-way analysis of variance (ANOVA) was used to analyze the statistical significances of the results, using Bonferroni post-hoc test for comparisons between individual groups. The data were considered significant at $P<0.05$.

3. Results

The GF cell cultures were challenged for 6 h with ascending concentrations of the supragingival or subgingival biofilm culture supernatants. Neither of these challenges increased toxicity compared to the control group (data not shown).

The effect of the two biofilms on the gene expression of three NLRP inflammasomes was then investigated. NLRP1 and NLRP3 mRNA expressions were ubiquitously detected in the cells, whereas NLRP2 expression was not detected in any group under the present experimental conditions. Neither the supragingival nor the subgingival biofilm affected NLRP1 expression (Figure 1). NLRP3 mRNA levels were not affected by the supragingival biofilm, but the highest concentration of

subgingival biofilm supernatant caused an 80% down-regulation, compared to the control (Figure 2). When the expressions of ASC (Figure 3), Caspase-1 (Figure 4) and AIM2 (Figure 5) were investigated, a similar pattern of regulation was observed among them. Specifically, the supragingival biofilm challenge caused a concentration-dependent increase in their gene expression. Interestingly, the concentration kinetics of the subgingival biofilm challenge were different. At lower concentrations, ASC, Caspase-1 and AIM2, mRNA levels were increased , followed by a decline to control levels by the highest supernatant concentration used.

As the activation of IL-1 cytokines is the down-stream target of inflammasomes, the gene expressions of IL-1β and IL-18, were also investigated. While IL-18 mRNA (Figure 7) was detected at lower levels than IL-1β (Figure 6), they were both regulated in a manner similar to AIM2, ASC and Caspase-1. Hence, the supragingival biofilm challenge caused a concentration-dependent increase, whereas the subgingival one had a biphasic effect, causing an increase in IL-1β and IL-18 gene expression at lower concentrations, but a decline to control levels at highest concentrations.

4. Discussion

This study identified that supragingival and subgingival biofilms exert differential effects on the gene expression of inflammasome complexes, indicating that these are recognised as PAMPs by the GF. The NLRP1 and NLRP2 inflammasomes do not appear to be of relevance to this experimental system, as the gene expression of the former is not affected by either biofilm challenge, whereas the latter is not expressed at all in GF. However, NLRP3 is responsive to oral biofilm challenge, as the gene expression of all of the components of this inflammasome, including ASC and

Caspase-1, are regulated. A differential response is observed between the supragingival and subgingival biofilm variants, denoting their different pathogenic potential for periodontal diseases. This is particularly highlighted by the drastically decreased NLRP3 gene expression by high concentrations of supragingival biofilm supernatants, also evident in ASC and Caspase-1 expressions. Hence, NLRP3 gene expression may have a central role within its own inflammasome complex, and a down-regulation could be detrimental for the expression of its other components (i.e. ASC and Caspase-1) in GF. Nevertheless, it is not yet clear if the observed regulations of inflammasome gene expression components reflect changes in the respective protein expressions and bioactivity.

Although inflammasomes are responsible for controlling IL-1 cytokine activation rather than gene expression, IL-1β and IL-18 followed a similar pattern to inflammasome expression, in the present experimental system in GF. These results denote that oral biofilms could concomitantly regulate the expression of inflammasomes and their associated cytokine targets. Earlier work using semi-quantitative qPCR has indicated that GF do not express IL-18, but express low levels of IL-1β mRNA [32]. The present study employing the more sensitive qPCR shows that IL-18 mRNA can also be detected in GF, albeit at lower levels than IL-1β. Of special note, the low levels of IL-1β protein production by GF [32, 33], along with the high cytokine-degradation effects of these *in vitro* biofilms [29], impose limitations in the study of IL-1β protein secretion in the present experimental system.

Less information is available on the recently discovered AIM2 inflammasome, which is so far known as a cytosolic double-stranded DNA sensor [22, 23] and a first non-NLR member forming an inflammasome scaffold [11]. The fact that AIM2 gene expression is regulated in the present model denotes that the cells may respond to the

bacterial DNA present in the biofilm culture supernatant, and that active bacterial invasion is not a pre-requisite for this process. Despite the recognition of different pathogen-associated molecular patterns, the NLRP3 and AIM2 inflammasomes share a common feature: apart from their homonymous scaffolds they both consist of Caspase-1 and the ASC adaptor [11].

The down-regulation of NLRP3 and AIM2 inflammasomes gene expressions and the concomitant decrease in IL-1β and IL-18 gene expressions by the subgingival biofilm challenge may be an important mechanism in the pathogenesis of periodontal disease. Indeed, inflammasome-mediated inhibition of IL-1 cytokine processing is considered a strategy by bacterial and viral pathogens to manipulate the local immune and inflammatory responses [34], in order to survive and prevail in the periodontal tissue environment [35]. In fact, the molecular cross-talk of *P. gingivalis* with the host enables it to evade the normal immune responses [36], potentially benefiting other co-habiting organisms [37]. *P. gingivalis* up-regulates NLRP3 [10, 38] but down-regulates ASC expression [10, 39] in monocytic cells, whereas it down-regulates NLRP3, but not ASC in gingival epithelial cells. [40]. Co-infection of host cells with *P. gingivalis* and other putative periodontal pathogens, decreases the IL-1-inducing capacity of the latter [41, 42], denoting an antagonistic capacity. This may well be the case in the present experimental system utilizing subgingival biofilms, where *P. gingivalis* is present.

The present study demonstrates that the NLRP3 and AIM2 inflammasomes are responsive to oral biofilm infection in GF. Supragingival and subgingival biofilms cause differential effects in the gene expression of these inflammasome components and their down-stream IL-1 targets. While typically an increase in inflammasome expression is commensurate with inflammatory events in periodontal disease, a

biphasic decrease could dampen the host immune responses, in favour of pathogen survival. This is in line with a potential tissue-gradient effect of periodontal pathogens on host cells [43]. Close to the biofilm-tissue interface where the innate immune mechanisms are affluent, the high concentration of bacterial virulence factors on site may down-play these critical host defence barriers. Deeper into the periodontal tissues, the lower bacterial component concentration could have a stimulatory effect on the inflammatory responses.

Acknowledgements

The authors would like to thank Mrs Elpida Plattner and Verena Osterwalder for their excellent technical assistance and Dr. Anders Johansson (Institute of Odontology, Umeå University, Sweden) for providing the GF. This study was supported by the authors' Institute.

References

[1] P.D. Marsh, Dental plaque: biological significance of a biofilm and community life-style, J Clin Periodontol, 32 Suppl 6 (2005) 7-15.

[2] S. Filoche, L. Wong, C.H. Sissons, Oral biofilms: emerging concepts in microbial ecology, J Dent Res, 89 (2010) 8-18.

[3] Z. Feng, A. Weinberg, Role of bacteria in health and disease of periodontal tissues, Periodontol 2000, 40 (2006) 50-76.

[4] H.A. Schenkein, Host responses in maintaining periodontal health and determining periodontal disease, Periodontol 2000, 40 (2006) 77-93.

[5] L.A. Christersson, J.J. Zambon, R.J. Genco, Dental bacterial plaques. Nature and role in periodontal disease, J Clin Periodontol, 18 (1991) 441-446.

[6] M. Kuboniwa, R.J. Lamont, Subgingival biofilm formation, Periodontol 2000, 52 (2010) 38-52.

[7] D.T. Graves, D. Cochran, The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction, J Periodontol, 74 (2003) 391-401.

[8] A.J. Delima, S. Karatzas, S. Amar, D.T. Graves, Inflammation and tissue loss caused by periodontal pathogens is reduced by interleukin-1 antagonists, J Infect Dis, 186 (2002) 511-516.

[9] S.P. Engebretson, J.T. Grbic, R. Singer, I.B. Lamster, GCF IL-1beta profiles in periodontal disease, J Clin Periodontol, 29 (2002) 48-53.

[10] N. Bostanci, G. Emingil, B. Saygan, O. Turkoglu, G. Atilla, M.A. Curtis, G.N. Belibasakis, Expression and regulation of the NALP3 inflammasome complex in periodontal diseases, Clin Exp Immunol, 157 (2009) 415-422.

[11] K. Schroder, J. Tschopp, The inflammasomes, Cell, 140 (2010) 821-832.

[12] Y. Ogura, F.S. Sutterwala, R.A. Flavell, The inflammasome: first line of the immune response to cell stress, Cell, 126 (2006) 659-662.

[13] O. Gross, H. Poeck, M. Bscheider, C. Dostert, N. Hannesschlager, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, J. Ruland, Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence, Nature, 459 (2009) 433-436.

[14] J. Ng, S.A. Hirota, O. Gross, Y. Li, A. Ulke-Lemee, M.S. Potentier, L.P. Schenck, A. Vilaysane, M.E. Seamone, H. Feng, G.D. Armstrong, J. Tschopp, J.A. Macdonald, D.A. Muruve, P.L. Beck, Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome, Gastroenterology, 139 (2010) 542-552, 552 e541-543.

[15] S. Mariathasan, D.S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W.P. Lee, Y. Weinrauch, D.M. Monack, V.M. Dixit, Cryopyrin activates the inflammasome in response to toxins and ATP, Nature, 440 (2006) 228-232.

[16] R.R. Craven, X. Gao, I.C. Allen, D. Gris, J. Bubeck Wardenburg, E. McElvania-Tekippe, J.P. Ting, J.A. Duncan, Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells, PLoS One, 4 (2009) e7446.

[17] J.J. Chae, H.D. Komarow, J. Cheng, G. Wood, N. Raben, P.P. Liu, D.L. Kastner, Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis, Mol Cell, 11 (2003) 591-604.

[18] J.J. Chae, G. Wood, S.L. Masters, K. Richard, G. Park, B.J. Smith, D.L. Kastner, The B30.2 domain of pyrin, the familial Mediterranean fever protein, interacts

directly with caspase-1 to modulate IL-1beta production, Proc Natl Acad Sci U S A, 103 (2006) 9982-9987.

[19] B. Faustin, L. Lartigue, J.M. Bruey, F. Luciano, E. Sergienko, B. Bailly-Maitre, N. Volkmann, D. Hanein, I. Rouiller, J.C. Reed, Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation, Mol Cell, 25 (2007) 713-724.

[20] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Mol Cell, 10 (2002) 417-426.

[21] E.D. Boyden, W.F. Dietrich, Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin, Nat Genet, 38 (2006) 240-244.

[22] T. Burckstummer, C. Baumann, S. Bluml, E. Dixit, G. Durnberger, H. Jahn, M. Planyavsky, M. Bilban, J. Colinge, K.L. Bennett, G. Superti-Furga, An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome, Nat Immunol, 10 (2009) 266-272.

[23] V. Hornung, A. Ablasser, M. Charrel-Dennis, F. Bauernfeind, G. Horvath, D.R. Caffrey, E. Latz, K.A. Fitzgerald, AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC, Nature, 458 (2009) 514-518.

[24] P.M. Bartold, L.J. Walsh, A.S. Narayanan, Molecular and cell biology of the gingiva, Periodontol 2000, 24 (2000) 28-55.

[25] A.S. Yazdi, S.K. Drexler, J. Tschopp, The role of the inflammasome in nonmyeloid cells, J Clin Immunol, 30 (2010) 623-627.

[26] S. Rosengren, H.M. Hoffman, W. Bugbee, D.L. Boyle, Expression and regulation of cryopyrin and related proteins in rheumatoid arthritis synovium, Ann Rheum Dis, 64 (2005) 708-714.

[27] L. Kolly, N. Busso, G. Palmer, D. Talabot-Ayer, V. Chobaz, A. So, Expression and function of the NALP3 inflammasome in rheumatoid synovium, Immunology, 129 (2010) 178-185.

[28] S. Shapiro, E. Giertsen, B. Guggenheim, An in vitro oral biofilm model for comparing the efficacy of antimicrobial mouthrinses, Caries Res, 36 (2002) 93-100.

[29] B. Guggenheim, R. Gmur, J.C. Galicia, P.G. Stathopoulou, M.R. Benakanakere, A. Meier, T. Thurnheer, D.F. Kinane, In vitro modeling of host-parasite interactions: the 'subgingival' biofilm challenge of primary human epithelial cells, BMC Microbiol, 9 (2009) 280.

[30] R. Gmur, B. Guggenheim, Antigenic heterogeneity of Bacteroides intermedius as recognized by monoclonal antibodies, Infect Immun, 42 (1983) 459-470.

[31] G.N. Belibasakis, N. Bostanci, A. Hashim, A. Johansson, J. Aduse-Opoku, M.A. Curtis, F.J. Hughes, Regulation of RANKL and OPG gene expression in human gingival fibroblasts and periodontal ligament cells by Porphyromonas gingivalis: a putative role of the Arg-gingipains, Microb Pathog, 43 (2007) 46-53.

[32] F. Tardif, G. Ross, M. Rouabhia, Gingival and dermal fibroblasts produce interleukin-1 beta converting enzyme and interleukin-1 beta but not interleukin-18 even after stimulation with lipopolysaccharide, J Cell Physiol, 198 (2004) 125-132.

[33] G.N. Belibasakis, A. Johansson, Y. Wang, C. Chen, T. Lagergard, S. Kalfas, U.H. Lerner, Cytokine responses of human gingival fibroblasts to Actinobacillus actinomycetemcomitans cytolethal distending toxin, Cytokine, 30 (2005) 56-63.

[34] D.J. Taxman, M.T. Huang, J.P. Ting, Inflammasome inhibition as a pathogenic stealth mechanism, Cell Host Microbe, 8 (2010) 7-11.

[35] D.R. Dixon, B.W. Bainbridge, R.P. Darveau, Modulation of the innate immune response within the periodontium, Periodontol 2000, 35 (2004) 53-74.

[36] J.L. Krauss, J. Potempa, J.D. Lambris, G. Hajishengallis, Complementary Tolls in the periodontium: how periodontal bacteria modify complement and Toll-like receptor responses to prevail in the host, Periodontol 2000, 52 (2010) 141-162.

[37] G. Hajishengallis, Porphyromonas gingivalis-host interactions: open war or intelligent guerilla tactics? Microbes Infect, 11 (2009) 637-645.

[38] M. Hamedi, G.N. Belibasakis, A.T. Cruchley, M. Rangarajan, M.A. Curtis, N. Bostanci, Porphyromonas gingivalis culture supernatants differentially regulate interleukin-1beta and interleukin-18 in human monocytic cells, Cytokine, 45 (2009) 99-104.

[39] D.J. Taxman, J. Zhang, C. Champagne, D.T. Bergstralh, H.A. Iocca, J.D. Lich, J.P. Ting, Cutting edge: ASC mediates the induction of multiple cytokines by Porphyromonas gingivalis via caspase-1-dependent and -independent pathways, J Immunol, 177 (2006) 4252-4256.

[40] O. Yilmaz, A.A. Sater, L. Yao, T. Koutouzis, M. Pettengill, D.M. Ojcius, ATP-dependent activation of an inflammasome in primary gingival epithelial cells infected by Porphyromonas gingivalis, Cell Microbiol, 12 (2010) 188-198.

[41] N. Bostanci, R.P. Allaker, G.N. Belibasakis, M. Rangarajan, M.A. Curtis, F.J. Hughes, I.J. McKay, Porphyromonas gingivalis antagonises Campylobacter rectus induced cytokine production by human monocytes, Cytokine, 39 (2007) 147-156.
[42] N. Bostanci, R. Allaker, U. Johansson, M. Rangarajan, M.A. Curtis, F.J. Hughes, I.J. McKay, Interleukin-1alpha stimulation in monocytes by periodontal bacteria: antagonistic effects of Porphyromonas gingivalis, Oral Microbiol Immunol, 22 (2007) 52-60.
[43] R.D. Pathirana, N.M. O'Brien-Simpson, E.C. Reynolds, Host immune responses to Porphyromonas gingivalis antigens, Periodontol 2000, 52 (2010) 218-237.

Figure Legends

Figure 1

Regulation of NLRP1 gene expression in GF in response to oral biofilm challenge.

GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h. The gene expression levels of NLRP1 were measured by qPCR analysis, normalized against the expression levels of 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference between the biofilm-challenged and control groups.

Figure 2

Regulation of NLRP3 gene expression in GF in response to oral biofilm challenge.

GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h. The gene expression levels of NLRP3 were measured by qPCR analysis, normalized against the expression levels of 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent

mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference between the biofilm-challenged and control groups.

Figure 3

Regulation of ASC gene expression in GF in response to oral biofilm challenge. GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h. The gene expression levels of ASC were measured by qPCR analysis, normalized against the expression levels of 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference between the biofilm-challenged and control groups.

Figure 4

Regulation of Caspase-1 gene expression in GF in response to oral biofilm challenge. GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h. The gene expression levels of Caspase-1 were measured by qPCR analysis, normalized against the expression levels of 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference between the biofilm-challenged and control groups.

Figure 5

Regulation of AIM2 gene expression in GF in response to oral biofilm challenge. GF cell cultures were challenged with ascending concentrations of supragingival or

subgingival biofilm supernatants for 6 h. The gene expression levels of AIM2 were measured by qPCR analysis, normalized against the expression levels of 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference between the biofilm-challenged and control groups.

Figure 6

Regulation of IL-1β gene expression in GF in response to oral biofilm challenge. GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h. The gene expression levels of IL-1β were measured by qPCR analysis, normalized against the expression levels of 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference between the biofilm-challenged and control groups.

Figure 7

Regulation of IL-18 gene expression in GF in response to oral biofilm challenge.
GF cell cultures were challenged with ascending concentrations of supragingival or subgingival biofilm supernatants for 6 h. The gene expression levels of IL-18 were measured by qPCR analysis, normalized against the expression levels of 18S rRNA (housekeeping gene). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference between the biofilm-challenged and control groups.

Figure 1

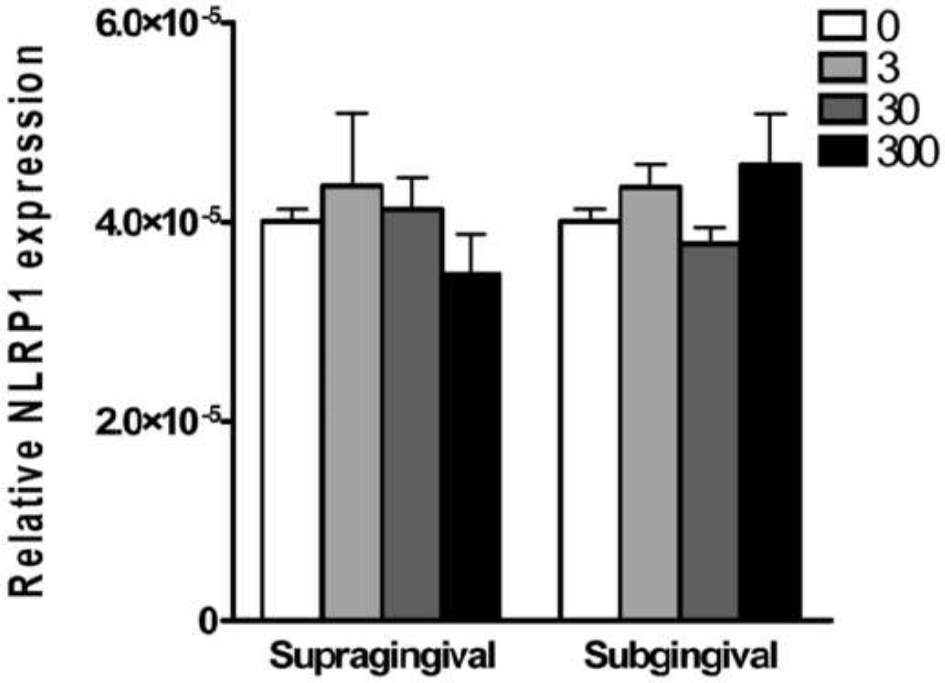


Figure 2

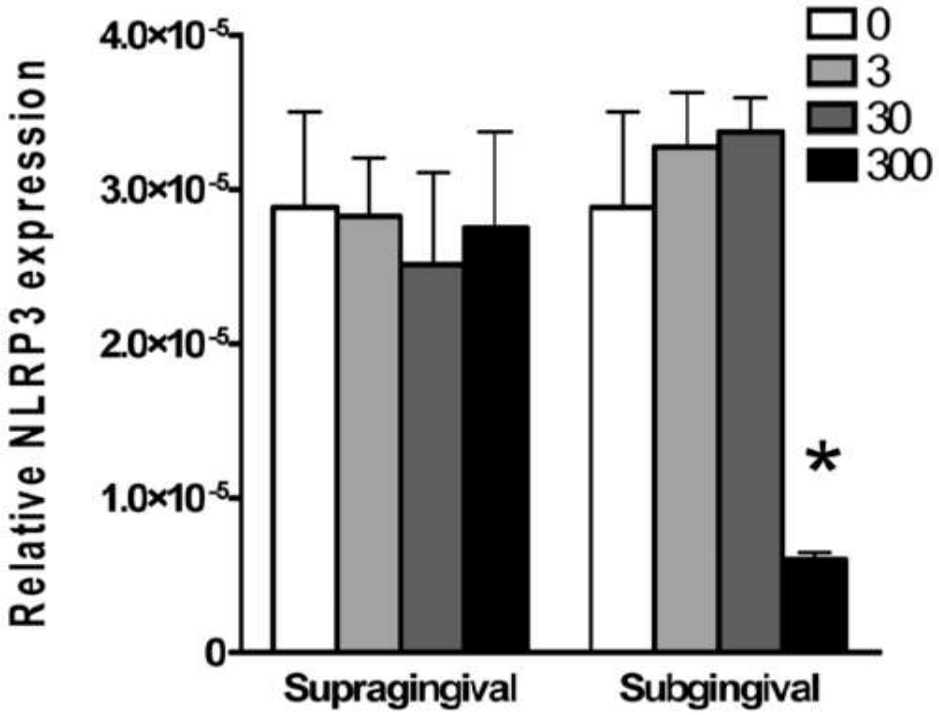


Figure 3

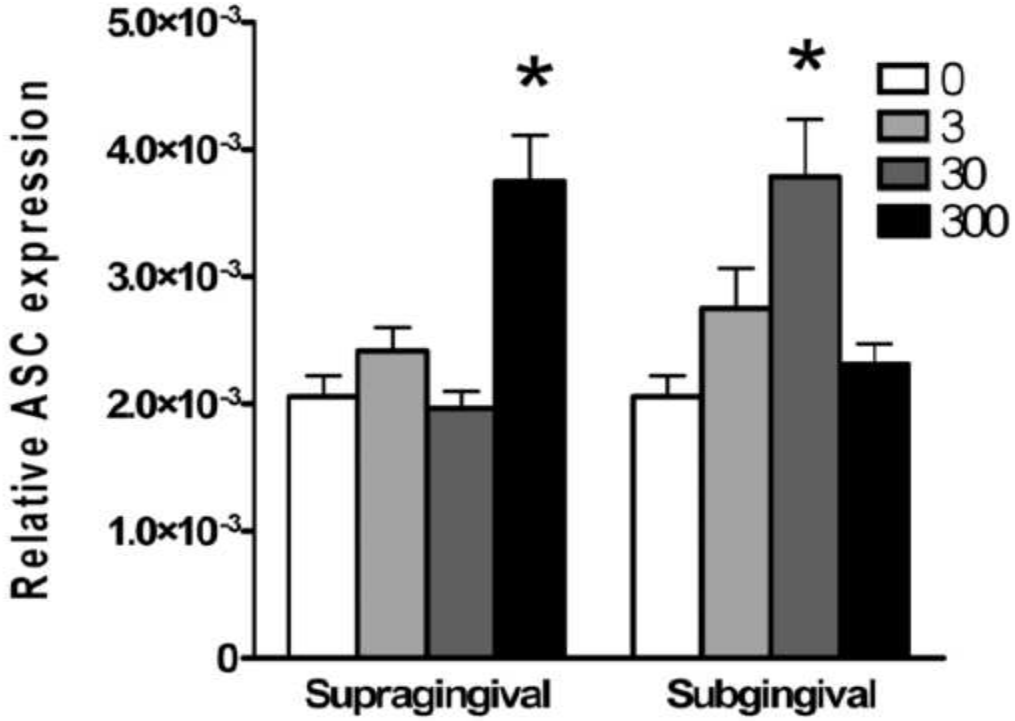


Figure 4

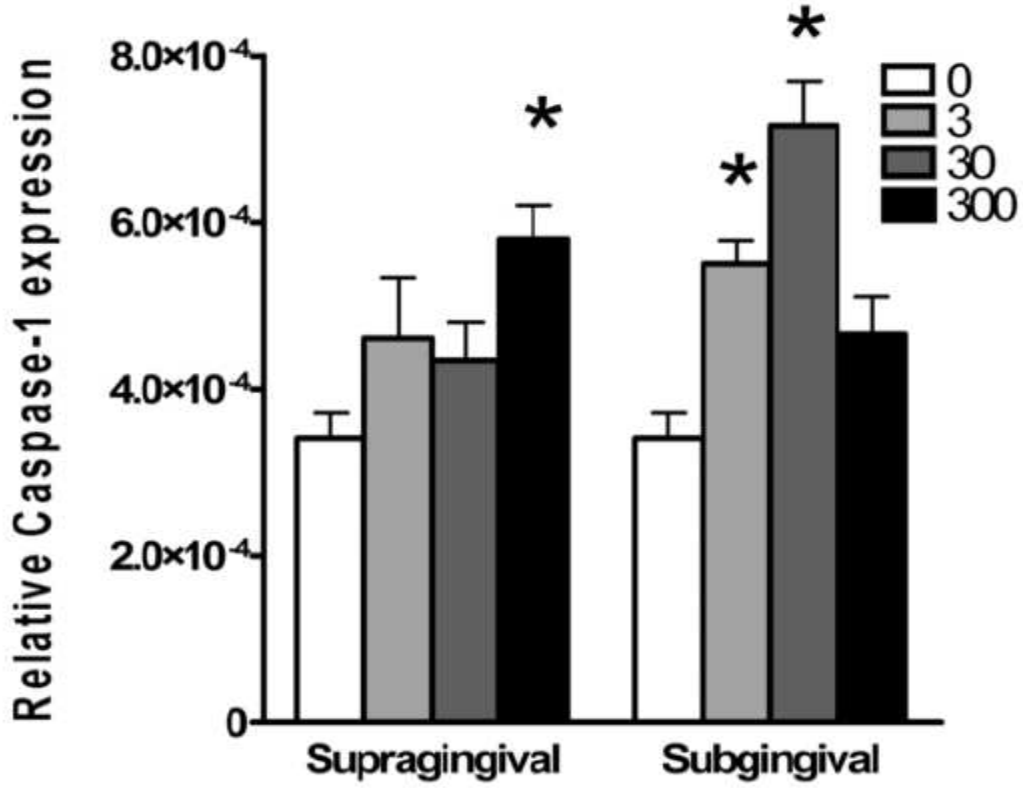


Figure 5

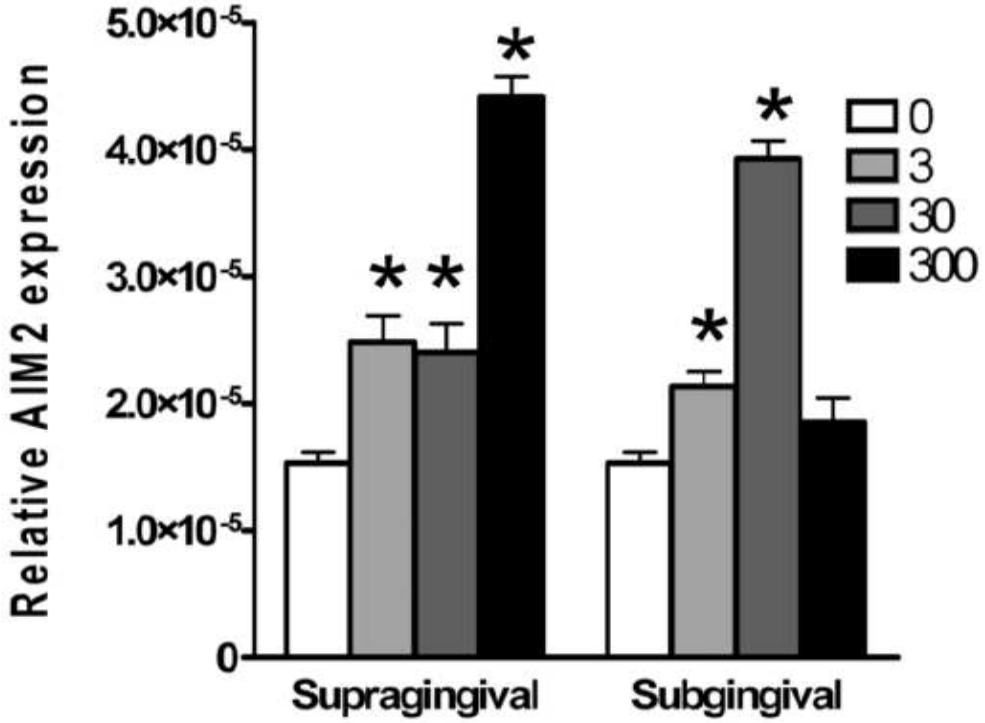


Figure 6

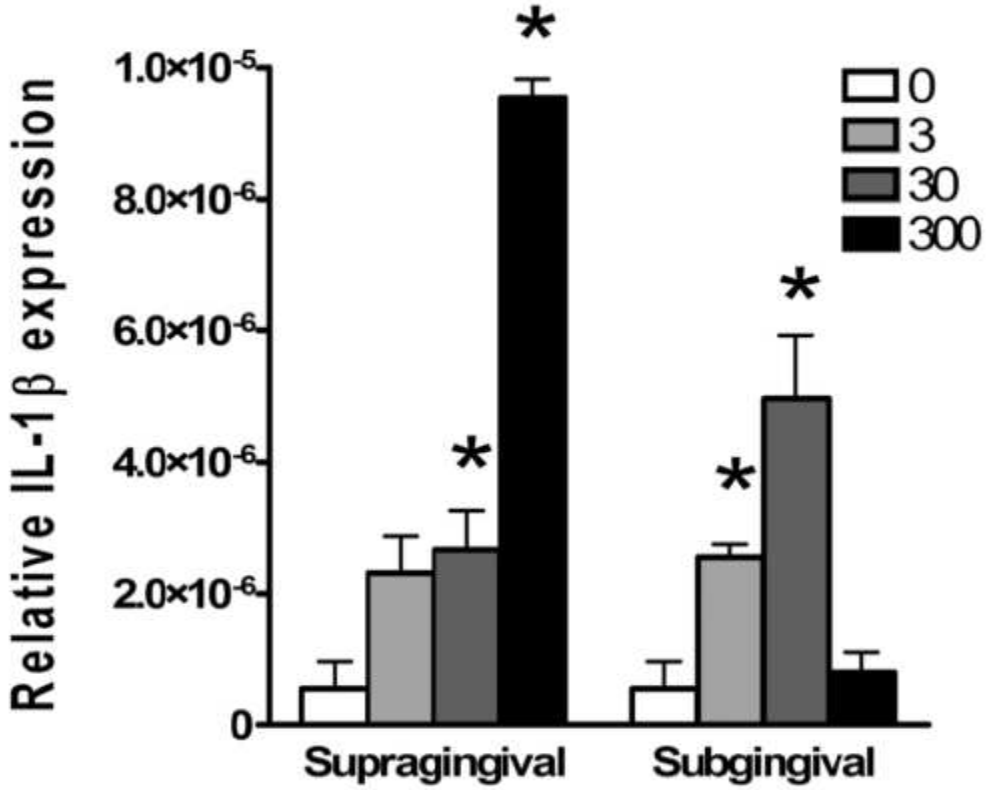
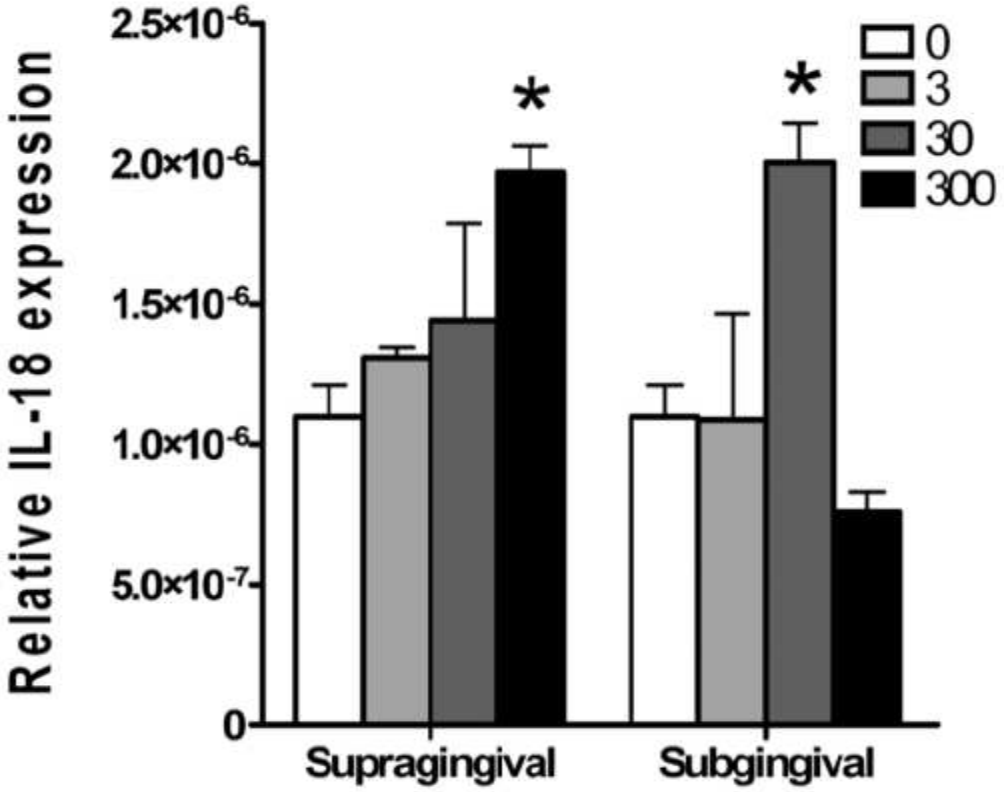


Figure 7


Relative IL-18 expression
2.5×10-6
2.0×10-6
1.5×10-6
1.0×10-6
5.0×10-7
0
*
*
0
3
30
300
Supragingival
Subgingival